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(54) Title: EXPANSION OF STEM CELLS IN LONG TERM BONE MARROW CULTURES BY NEUTRALIZATION OF TGF-β

(57) Abstract

A method for expanding growth of hematopoietic stem cells in culture includes a step of admixing to the culture a TGF-β antagonist such as a soluble TGF-β receptor or a TGF-β-neutralizing antibody. Also, treating an animal includes steps of admixing a TGF-β antagonist to a culture of bone marrow cells to stimulate production of hematopoietic stem cells in the culture, and thereafter administering hematopoietic cells so produced to the animal. Also, a method for treating an animal includes steps of administering a TGF-β antagonist to the animal.

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EXPANSION OF STEM CELLS IN LONG TERM BONE MARROW CULTURES BY NEUTRALIZATION OF TGF-β

Background of the Invention

i. Technical Field

This invention relates to regulation of hematopoiesis, and particularly to regulating production of hematopoietic stem cells.

ii. Background Art

The transforming growth factor betas (TGF-β) are a family of cytokines for which a major function is inhibition of cell growth and differentiation. There is evidence that TGF-β inhibits cell proliferation by extending or arresting the G1 phase of the cell cycle (A.B. Roberts *et al.* (1985), *Proc. Nat'l. Acad. Sci. USA*, Vol. 82, pp. 119 ff.). TGF-β has been shown to be a negative regulator of hematopoiesis in long term bone marrow (LTBM) cultures. Addition of picomolar concentrations of TGF-β to LTBM inhibits hematopoiesis with the greatest effects seen on the early granulo-monocytic lineage and erythroid lineages and lesser effects seen on lymphocytic lineages (S.I. Hayashi *et al.* (1989), *Blood*, Vol. 74, No. 5, pp. 1711 ff.; J.A. Carlino *et al.* (1992), *Exp. Hematol.*, Vol. 20, pp. 943 ff.). Inhibition of megakaryocytic progenitors has also been demonstrated with TGF-β treatment (Z.C. Han *et al.* (1991), *Int. Jour. Hematol.*, Vol. 54, No. 1, pp. 3 ff.; T. Ishibashi *et al.*, (1987), *Blood*, Vol. 69, pp. 1737 ff.).

TGF-β has been shown to act directly on an enriched population of high proliferation multipotential

hematopoietic progenitors from bone marrow (J.R. Keller *et al.* (1990), *Blood*, Vol. 75, pp. 596 ff.). In addition, there is also evidence that TGF-β also affects the mesenchymal stromal elements of these cultures (J.D. Cashman *et al.* (1990), *Blood*, Vol. 75, No. 1, pp. 96 ff.).

Immunohistochemical localization of high levels of TGF-β in bone marrow and fetal liver as well as detection of TGF-β mRNA and protein in established LTC suggest an intrinsic role of TGF-β in regulating these cells (L.R. Ellingsworth *et al.* (1986), *Jour. Biol. Chem.*, Vol. 261, pp. 12362 ff.; C.J. Eaves *et al.* (1991), *Blood*, Vol. 78, No. 1, pp. 110 ff.). Recent studies have shown that addition of antibodies to TGF-β or TGF-β anti-sense DNA to LTC prolongs or reactivates the number of colonies formed by multilineage, early erythroid and granulo-monocytic progenitors but not late progenitors (C.J. Eaves *et al.* (1991), *supra*; J. Hatzfield *et al.* (1991), *Jour. Exp. Med.*, Vol. 174, pp. 925 ff.).

Summary of the Invention

We have discovered that endogenously produced TGF-β has a role in hematopoietic growth regulation, and that addition of an agent that neutralizes the activity of TGF-β types 1, 2, and 3 (that is, an agent that operates as a TGF-β antagonist) can stimulate production of hematopoietic stem cells *in vitro* and *in vivo*.

We have demonstrated, for example, that treating Dexter-type long-term murine bone marrow cultures with a monoclonal antibody that neutralizes the biological activity of TGF-β types 1, 2 and 3 can provide for substantially expanded growth of hematopoietic stem cells in such cultures.

We have further discovered that long term survival of lethally-irradiated animals can be improved by administering to the animals bone marrow cells that have been cultured in the presence of an effective amount of an agent that neutralizes the biological activity of TGF-β types 1, 2 and 3 (that is, an agent that operates

as a TGF-β antagonist) to stimulate production of hematopoietic stem cells in the culture. We have demonstrated, for example, improvement in long term survival of lethally-irradiated animals by administering to the animals bone marrow cells cultured in the presence of an effective amount of a monoclonal antibody that neutralizes the biological activity of TGF-β types 1, 2 and 3, or in the presence of an effective amount of a soluble form of a TGF-β receptor, to stimulate production of hematopoietic stem cells in the culture.

We have further discovered that long term survival of immunocompromised animals can be improved by administrating to the animal an effective amount of an agent that neutralizes the biological activity of TGF-β types 1, 2 and 3 (that is, an agent that operates as a TGF-β antagonist). We have demonstrated, for example, improvement in long term survival of animals that have been treated with a lethal dose of a chemotherapeutic drug (*e.g.*, 5-fluorouracil) by administration of an effective amount of a monoclonal antibody that neutralizes the biological activity of TGF-β types 1, 2 and 3 or administration of an effective amount of a soluble form of a TGF-β receptor to the animal at the time of treatment with the chemotherapeutic drug.

iii. Disclosure of Invention

In one general aspect the invention features a method for expanding growth of hematopoietic stem cells in culture, including a step of admixing a TGF-β antagonist to the culture.

The term "expanding growth of hematopoietic stem cells" is used herein to mean an increase in the numbers of hematopoietic stem cells in the culture.

In some embodiments, the TGF-β antagonist includes a soluble TGF-β receptor; in other embodiments the TGF-β antagonist includes an antibody or a combination of antibodies, more preferably a monoclonal antibody or a combination of monoclonal antibodies, that neutralizes

TGF-β types 1, 2, and 3. A "TGF-β antagonist", as that term is used herein, is a substance that inhibits, or a combination of substances that together inhibit, the biological activity of TGF-β. Similarly, a molecule, such as an anti-TGF-β antibody, that "neutralizes" TGF-β, as that term is used herein, is a substance that inhibits the *in vitro* biological activity of TGF-β. A most preferred TGF-β antagonist is a monoclonal antibody that cross-reacts with and neutralizes TGF-β types 1, 2, and 3. By way of example, one such TGF-β antagonist is a monoclonal antibody ("1D11.16") produced by a murine hybridoma cell line, 1D11.16, as described in copending U.S. patent application Serial No. 07/759,109, filed September 6, 1991, which is hereby incorporated herein by reference in its entirety.

In another general aspect, the invention features a method for treating an animal, by culturing bone marrow cells *in vitro*, admixing a TGF-β antagonist with the culture to stimulate production of hematopoietic stem cells, and administering cells from the culture to the animal.

In preferred embodiments the bone marrow cells are cells that were derived from an animal of the same species as the animal to be treated, and more preferably are cells that were harvested from the animal to be treated.

In another general aspect, the invention features a method for treating an animal, by administering a TGF-β antagonist to the animal.

Description of Preferred Embodiments

Dexter cultures have been treated with a monoclonal antibody, 1D11.16, which neutralizes the biological activity of TGF-β types 1, 2 and 3, or with a control antibody. The composition and cellularity of the nonadherent cell populations in these cultures were assessed weekly. Treatment with anti-TGF-β Ab resulted in a 5-9 fold increase in nonadherent cells in the cultures as compared to either the control or untreated cultures by week four. The majority of these cells were granulocyte/ macrophage lineage cells as assessed by histologic and flow cytometric analysis. There was also a significant increase in megakaryocytes in cultures treated with anti-TGF-β Ab. Stem cell analysis, using a CFU-S assay, combining both the adherent and nonadherent populations from either 4 or 6 week cultures, showed that there are an equivalent number of hematopoietic stem cells per 10^6 cells regardless of antibody treatment. Therefore, anti-TGF-β antibody treated cultures contained at least 5 times as many stem cells as control cultures. Finally, kinetics studies show that the presence of anti-TGF-β antibody is required from the onset of culture to produce these effects. These results suggest that TGF-β is involved in normal growth regulation of bone marrow hematopoietic cells. By addition of a neutralizing antibody, the normal TGF-β negative growth signal is disrupted, allowing expanded growth of several cell populations.

In the present studies we have treated Dexter type LTBM cultures with either an antibody which neutralizes TGF-β1, TGF-β2 and TGF-β3 or with a control antibody. We have examined the resulting cell populations histologically and by flow cytometric analysis. By disrupting the negative growth control exerted by endogenous TGF-β on hematopoietic progenitors a dramatic nonadherent cell outgrowth is seen. Phenotypically, most of the nonadherent cells that exhibit increased growth in neutralizing antibody treated cultures are those of the

monocyte and granulocyte lineages and megakaryocytes. Analysis of both adherent and nonadherent cells indicates a similar increase in CFU-S stem cells.

iv. Brief Description of Drawings

Fig. 1 is a histogram showing results of treatment of cells in Dexter long term bone marrow culture with an anti-TGF-β antibody. Nonadherent cells from triplicate cultures were mixed and counted at weekly intervals, beginning one week after initiation of the culture (solid bars) and continuing for 5 weeks (week 2, dark crosshatched bars; week 3, shaded bars; week 4, light crosshatched bars; week 5, open bars). Cell counts in cultures treated with anti-TGF-β monoclonal antibody were at a maximum 8 times as great at 4 weeks as in cultures receiving a control antibody, or in cultures receiving no treatment. Data in the Fig. represent 5 separate experiments.

Fig. 2 is a histogram showing results of treatment of cells in Dexter long term bone marrow culture with various quantities of anti-TGF-β antibody. Nonadherent cells from triplicate cultures were mixed and counted at weekly intervals, beginning one week after initiation of the culture. Cell counts increased in cultures treated with anti-TGF-β antibody (5 μg/ml, light shaded bars; 10 μg/ml, light crosshatched bars; 25 μg/ml, clear bars; 50 μg/ml, dark shaded bars) in dose dependent fashion. Cell numbers in untreated cultures (solid bars) or cultures treated with 50μg/ml control antibody (dark crosshatched bars) increased little during this culture period. Data in Fig. 2 represent 3 separate experiments.

Fig. 3 is a histogram showing results of initiating treatment of cells in Dexter long term bone marrow cultures with anti-TGF-β antibody at the time of initiation of the cultures or at various times (1, 2, or 3 weeks) following initiation of the cultures. Nonadherent cells from triplicate cultures were mixed and counted. Cultures were set up untreated or with 25 μg/ml

control antibody (crosshatched bars) or anti-TGF-β antibody (solid bars). Nonadherent cell growth increased only in cultures to which anti-TGF-β antibody was added at initiation.

Figs. 4.1 - 4.3 are prints of photomicrographs (10X magnification, Giemsa stain) showing samples of nonadherent cell populations cytospun onto glass slides from 5 week cultures. A modest yield of maturing granulocyte/macrophage lineage cells and occasional erythroid elements appear untreated cultures (Fig. 4.1) and in cultures treated with a control antibody (Fig. 4.2). A markedly higher yield of granulocyte/macrophage lineage, and numerous megakaryocytes (*) appear in cultures treated with anti-TGF-β antibodies (Fig. 4.3).

Figs. 5.1 - 5.3 are prints of photomicrographs showing stromal layers of 3 week old Dexter cultures. Few hematopoietic progenitor cells are attached to the adherent layer in untreated cultures (Fig. 5.1). A slightly greater number of hematopoietic progenitor cells are attached to the adherent layer in culture treated with a control antibody (MOPC 21C) (Fig. 5.2). A markedly greater number of progenitor cells are attached in hematopoietic islands in the adherent layer in cultures treated with anti-TGF-β antibody (Fig. 5.3).

Fig. 6 is a histogram showing the number of Day 14 CFU-S colonies induced by 2 X 10^5 cells is the same for all Dexter culture conditions tested (untreated; treated with control antibody; treated with anti-TGF-β antibody). Data shown represent three experiments in which similar results were seen.

v. Best Mode of Carrying out the Invention

The operation of the invention will now be illustrated by way of examples employing a mouse animal model system.

General techniques.

Animals Balb/c mice (Jackson Laboratories), 6-8 weeks old, were used in all the examples.

Antibodies Monoclonal antibodies 1D11.16 and MOPC 21C and 1410 KG7 were purified over a protein A column (J.W. Goding *et al.* (1976), *Jour. Immunol. Methods*, Vol. 42, pp. 17 ff.) from ascites fluid. SDS PAGE gels were run to assess purity (U.K. Laemmli (1970), *Nature*, Vol. 227, pp. 680 ff.).

1D11.16 is an IgG1 antibody which neutralizes the biological activity of TGF-β1, TGF-β2 and TGF-β3, was produced in Balb/c mice immunized with TGF-β2 generally as described in J.R. Dasch *et al.* (1989), *Jour. Immunol.*, Vol. 142, No. 5, pp. 1536 ff. MOPC 21C, a murine IgG1 plasmacytoma of unknown specificity, was obtained from ATCC for use as a control antibody (P.M. Knopf *et al.* (1973), *Eur. Jour. Immunol.*, Vol. 3, pp. 251 ff.). 1410 KG7, also an IgG1 monoclonal antibody reactive with human gamma heavy chains, was obtained from ATCC for use as an alternate control antibody. Antibodies used in flow cytometric analysis were as follows: anti-mouse IgG-PE (Southern Biotechnology), anti-mouse thy 1-FITC (Becton Dickinson), anti-MAC 1 (anti-mouse macrophage; Boehringer Mannheim), 8C5 (anti mouse granulocyte; supplied by DNAX corp.), and anti-rat IgG-PE (Southern Biotechnology).

Long term bone marrow cultures Dexter type LTBM cultures were initiated and maintained using techniques generally as described by T.M. Dexter *et al.* (1977), *Jour. Cell Physiol.*, Vol. 91, pp. 335 ff. Briefly, cultures were initiated with freshly isolated bone marrow and triplicate cultures were seeded with 1.5×10^7 cells in 8 ml of culture medium in 25 cm^2 tissue culture flasks. These were maintained in alpha MEM supplemented with 20%

horse serum (Gibco), Penicillin, Streptomycin and 10^{-7} M hydrocortisone sodium succinate (Elkins-Sinn, Inc). Every 7 days half of the spent media was removed and replaced with fresh media. Antibodies were added at the start of culture unless otherwise noted. Cultures were refed weekly by removal of 4 ml of medium, followed by addition of 4 ml of medium supplemented with antibody at the original concentration. In a kinetics experiment antibody was added to cultures starting at culture initiation and at 1, 2, and 3 weeks after initiation.

Histology Nonadherent cells were adhered to glass slides using a cytospin, and stained on the slides with a Wright-Giemsa stain. Cell populations that were adherent in culture were stained with a Wright-Giemsa stain directly in the tissue culture flask.

Assay of colony forming units per spleen CFU-S assays were performed generally as described in J.E. Till *et al.* (1961), *Radiat. Res.*, Vol. 14, pp. 213 ff. Briefly, Balb/c mice were irradiated with 600 R and groups of 10 mice each were reconstituted by tail vein injection either with 2×10^5 cultured cells or with 1×10^5 freshly isolated bone marrow. Cultured cells included both the nonadherent and adherent fractions of either 4 or 6 week old Dexter-type bone marrow cultures that had been incubated in the presence of anti-TGF-β antibody or of control antibody, or without antibody. Fourteen days after reconstitution spleens were removed and the numbers of colonies per spleen were counted.

Flow cytometric analysis Populations of nonadherent cells were phenotypically analyzed using a Facstar fluorescence activated cell sorter (Becton Dickinson). The cells were stained at a concentration of 1 μg antibody per 1×10^6 cells, on ice, for 30 minutes. The cells were washed and 10,000 cells were analyzed for each sample.

Anti-TGF-β treatment stimulates growth of nonadherent cells in Dexter cultures

In a first example, we demonstrated the consequences of inhibiting serum derived and endogenous TGF-β in Dexter type long term bone marrow cultures with a monoclonal antibody, 1D11.16, that neutralizes the activity of TGF-β1, TGF-β2 and TGF-β3. As Fig. 1 shows, continual treatment of such cultures with anti-TGF-β starting at the onset of culture induces an increase in nonadherent cell numbers appearing as early as one week after initiation culture, with maximal response appearing at 4 weeks. In five separate experiments there was an average increase of 12 ± 3 fold in anti-TGF-β treated cultures as compared to control cultures.

Effects of anti-TGF-β in Dexter cultures are dose dependent

In a second example, we demonstrated the effects of administering anti-TGF-β antibody to the cultures in various amounts (5, 10, 25 or 50 μg/ml) at the time of initiating the cultures. As Fig. 2 shows, weekly counts of nonadherent cells showed a dose dependent increase in cell numbers that was maximal at week 4. Cultures treated with the same concentrations of control antibody (data from administration of 50μg/ml is shown in Fig. 2 as representative). Concentrations of anti-TGF-β Ab as low as 5 μg/ml displayed stimulatory effects on nonadherent cell growth after several weeks of culture, suggesting that very little TGF-β is needed to regulate cell proliferation in this system.

TGF-β activity must be neutralized at the time of initiation of the culture to induce the increase in nonadherent cell growth

In a third example, 25 μg/ml neutralizing antibody wee administered to the cultures at the time of initiation of the culture, or at 1, 2, or 3 weeks after initiation. As Fig. 3 shows, antibody must be present at the start of culture to induce an increase in the nonadherent cell populations. Nonadherent cell counts in

4 week old cultures treated with anti-TGF-β showed a six-fold increase in cell number when antibody was introduced at culture initiation and a three fold increase when antibody was introduced after 1 week of incubation. No effect was seen if antibody was added later than 1 week after culture initiation.

Histological evaluation of nonadherent and adherent cell populations from treated cultures shows changes in granulocyte/ macrophage precursors, megakaryocytes and stromal layers

Cytospins of nonadherent cell populations from these cultures show an increase in cells of the granulocyte/macrophage lineage, and an appearance of numerous megakaryocytes in anti-TGF-β treated cultures over time (Figs. 4.1 - 4.3). Differential counts of cytospins from 5 week cultures revealed a shift in the anti-TGF-β treated cultures to a more granulocytic population (80 %), and fewer macrophages (15 %). In addition, there was a significant increase in megakaryocytes as compared to untreated cultures or those treated with control antibody. This comparison is made to control cultures that had nearly the same number of granulocytes and macrophages and almost no detectable megakaryocytes (see Table, following). There are marked differences in the stromal layers of cultures treated with anti-TGF-β as compared to controls. Significantly fewer hematopoietic progenitor cells were attached to the adherent layer in untreated cultures and in cultures treated with control antibody than in the anti-TGF-β treated cultures (Figs. 5.1 - 5.3).

Table

Differential Cell Counts of Nonadherent Cells After Anti-TGF-β Treatment for 5 Weeks

	Granulocyte Lineage	Macrophage Lineage	Megakaryocyte
Untreated	42 (57%)	31 (43%)	0 (0%)
Control Ab	43 (52%)	40 (48%)	0 (0%)
Anti-TGF-β	113 (80%)	25 (17%)	4 (3%)

Phenotypic analysis using flow cytometry confirms that the nonadherent cell population is of the granulocyte/macrophage lineage

Flow cytometric analysis of the nonadherent cell population was carried out weekly for six weeks after initiation of the culture. Cells were examined phenotypically using antibodies to Thy 1.2, murine Ig, murine granulocytes (8C5) and murine macrophages (MAC 1). Antibodies to Thy 1.2 and mouse Ig stained essentially as background. In contrast, a large percentage of the cells were of the macrophage/granulocyte lineage, as they stained with both MAC 1 and 8C5 in samples from all culture treatments. In the anti-TGF-β treated cultures there was an increase, at later times, in the number of cells staining with 8C5 that displayed more side scatter. These may represent the increased megakaryocyte population noted in these cultures during histological evaluation (data not shown in the Figs.).

Combined adherent and nonadherent cells from anti-TGF-β treated cultures had increased ability to induce colonies in a CFU-S assay

The potential of these Dexter long term bone marrow cells to repopulate the hematopoietic compartment *in vivo* was tested using a CFU-S assay. Adherent and nonadherent

populations from either 4 or 6 week old cultures were mixed and injected into lethally irradiated, syngeneic mice. As Fig. 6 shows, there were no differences in the numbers of colonies per spleen in day 14 CFU-S when 2×10^5 cells from untreated cultures, control antibody treated cultures, or anti-TGF-β treated cultures were used to reconstitute these mice. No statistically significant differences in the numbers of survivors appeared between the different groups. There was 20% survival in the saline control group, 90% survival in the group receiving freshly isolated bone marrow and 50% survival in all groups receiving cultured cells irrespective of treatment of the culture (data not shown). As there were 5 to 10 fold more cells in the anti-TGF-β treated cultures as compared to the controls it is clear that inhibition of TGF-β in these cultures permitted substantial early expansion of the hematopoietic progenitor cell population.

vi. Industrial Applicability

The method of the invention can be used for greatly expanding the numbers of a variety of types of hematopoietic stem cells in culture, without substantially altering the cell types in the culture. The invention thus provides means for obtaining hematopoietic stem cells in high number in culture.

As described in a further example below, the invention provides for means for improving or restoring hematopoietic function in an animal. For example, an animal such as a human patient may be subject to a form of medical treatment, such as radiation treatment or chemotherapy, that effectively destroys hamatopoietic function. In one approach, bone marrow cells are removed prior to the medical treatment and brought into culture; the TGF-β antagonist is then admixed to the culture to expand the numbers of the hematopoietic stem cells, according to the method of the invention. Following the

medical treatment, the hematopoietic stem cells from the culture are returned to the patient.

Additionally, as described in a still further example below, a TGF-β antagonist can according to the invention be administered directly to an animal that is in need of improvement or restoration of hematopoiesis, in an amount effective to stimulate expansion of hematopoietic stem cells *in vivo*.

Numerous studies have shown TGF-β to be a potent modulator of hematopoietic stem cell growth and differentiation. Although there are conflicting reports as to specific cytokine/TGF-β interactions, the more primitive stem cells such as multipotential progenitor cells (CFU-GEMM) and high proliferative potential colony forming cells (HPP-CFC) have consistently been shown to be growth inhibited by TGF-β (*see, e.g.*, J.D. Cashman *et al.* (1990), *supra*; S.E.W. Jacobsen *et al.* (1991), *Blood*, Vol. 78, No. 9, pp. 2239 ff.; J.R. Keller *et al.* (1989), *Jour. Cell. Biochem.*, Vol. 39, pp. 175 ff.; K. Kishi *et al.* (1989), *Leukemia*, Vol. 3, No. 10, pp. 687 ff.; J.R. Keller *et al.* (1992), *Int. Jour. Cell Clon.*, Vol. 10, pp. 2 ff.; W. Piacibello *et al.* (1991), *Exp. Hematol.*, Vol. 19, pp. 1084 ff.). More mature hematopoietic stem cells, such as those stimulated to form colonies in response to G-CSF, M-CSF and GM-CSF, have been reported to be either stimulated or unaffected by treatment with TGF-β (*see, e.g.*, S.E.W. Jacobsen *et al.* (1991), *supra*; J.R. Keller *et al.* (1989), *supra*; J.R. Keller *et al.* (1992), *supra*; W. Piacibello *et al.* (1991), *supra*; K. Fan *et al.* (1992), *Blood*, Vol. 79, No. 7, pp. 1679 ff.; W.C. Hooper *et al.* (1991), *Leukemia Res.*, Vol. 15, No. 4, pp. 179 ff.; M. Sargiacomo *et al.* (1991), *Ann. N.Y. Acad. Sci.*, Vol. 628, pp. 84 ff.; I. Bursuker *et al.* (1992), *Exp. Hematol.*, Vol. 20, pp. 431 ff.).

The Dexter-type long term bone marrow culture system provides a model which has both bone marrow stroma and hematopoietic stem cells in various stages of

differentiation. After the formation of an adequate stromal layer, Dexter cultures support the growth of hematopoietic stem cells of the granulocyte, monocyte/macrophage, and megakaryocyte lineages and of HPP-CFC. In addition, cells in these cultures are capable of producing colonies in the spleens of irradiated animals (CFU-S) (*see, e.g.,* P.J. Quesenberry *et al.* (1989), *Ann. N.Y. Acad. Sci.*, Vol. 554, pp. 116 ff.). Exogenous TGF-β has been shown to have a profound effect on the ability of these cultures to maintain growth of any of these hematopoietic lineages. This effect is mediated through direct interaction with both the stromal microenvironment and the hematopoietic stem cells (*see,e.g.,* S.I. Hiyashi *et al.* (1989), *supra*; Z.C. Han *et al.* (1991), *supra*; P.W. Kincade (1990), *Adv. Cancer Res.*, Vol. 54, pp. 235 ff.; D.J. Kuter *et al.* (1992), *Blood*, Vol. 79, No. 3, pp. 619 ff.; F.W. Ruscetti *et al.* (1991), *Ann. N.Y. Acad. Sci.*, Vol. 628, pp. 31 ff.).

C.J. Eaves *et al.* (1991), *supra*, have demonstrated constitutive levels of TGF-β mRNA in the stromal layer of human long term bone marrow cultures and have shown that TGF-β bioactivity is detectable in culture supernatants. Antibodies to TGF-β altered the cell cycling of the primitive progenitors in these cultures by prolonging or reactivating their period in S phase. J. Hatzfield *et al.* (1991), *supra* showed that colony formation by multilineage early, but not late, erythroid and granulocyte progenitors was increased by the addition of an anti-sense sequence of TGF-β or antibodies to TGF-β. These studies demonstrate that TGF-β is present in long term bone marrow cultures and that neutralization of TGF-β activity affects the cellular makeup of these cultures.

We have demonstrated that treating murine Dexter cultures with a neutralizing antibody to TGF-β1, TGF-β2 and TGF-β3 results in growth stimulation of the stromal elements, early hematopoietic progenitors capable of

producing day 14 CFU-S, more mature cells of the granulocyte/macrophage lineage, and megakaryocytes. This effect may be mediated through the stromal layer, as kinetic studies revealed that neutralization of endogenous TGF-β must occur during the first week of culture when the stromal layer is forming. Later addition of anti-TGF-β did not release the progenitor cells from normal growth controls and no increase in cell numbers was seen.

Treatment with anti-TGF-β did not phenotypically alter nonadherent cell types supported by the Dexter cultures. Granulocyte and monocyte/macrophage lineage cells were identified, both histologically and by flow cytometric analysis, to be the primary cell types present with or without anti-TGF-β treatment. The major observed effect of abrogating TGF-β in these cultures is extensive proliferation. This resulted in up to 10 fold greater numbers of nonadherent, late progenitors of the granulocyte and macrophage lineages and a more extensive stromal layer with many more immature hematopoietic stem cells bound than seen in control cultures. Megakaryocyte precursors have been shown to be highly sensitive to inhibition by TFG-β (*see, e.g.,* Z.C. Han *et al.* (1991), *supra*; D.J. Kuter *et al.* (1992), *supra*); and neutralization of TGF-β induced at least a five fold increase in the number of megakaryocytes in our examples.

TGF-β has been shown to be able to inhibit cell cycling of CFU-S and CFU-S colony formation (*see,* e.*g.*, A. Migdalska *et al.* (1991), *Growth Factors*, Vol. 4, pp. 239 ff.; G.J. Graham *et al.* (1990), *Prog. Growth Factor Res.*, Vol. 2, pp. 181 ff.; J. Hampson *et al.* (1991), *Exp. Hematol.*, Vol. 19, pp. 245 ff.). We have shown that cells in anti-TGF-β treated Dexter cultures would have an increased capacity to repopulate an *in vivo* hematopoietic compartment. A day 14 CFU-S assay showed that the number of CFU-S colonies increased in direct proportion to the increase in the total cell number in

the anti-TGF-β treated cultures. This effect was seen only when both the adherent and nonadherent populations were used for reconstitution and no CFU-S were seen when only the nonadherent population was used (data not shown). This provides further evidence that the more immature, early progenitors, which are bound to the stromal layers of these cultures, contain cells that are normally inhibited from proliferating owing to the influence of TGF-β. As we have demonstrated, this population is allowed to expand in the presence of anti-TGF-β, allowing for an increased number of CFU-s to develop.

The animal model used in our examples provides an understanding of TGF-β suppression in a well defined bone marrow culture system. Neutralization of TGF-β under Dexter culture conditions induced release of a variety of cells from normal growth control. The types of cells found in these cultures do not appear to be altered in spite of marked expansion of cell numbers. This suggests that the role of TGF-β in hematopoietic regulation of bone marrow is primarily on growth control rather than that of differentiation.

Treatment with TGF-β antagonists stimulates production of hematopoietic stem cells in vitro and in vivo

In a further example, we collected bone marrow from Balb/c mice and grew the cells *in vitro* for 2 weeks in the presence of 0.1-1000 mg/ml of an anti-TGF-β mAb or 0.01-10 mg/ml of a soluble form of a TGF-β receptor. We then transplanted adherent and nonadherent cells from these cultures into groups of 10 lethally irradiated animals, and assessed their long term survival. A dose-dependent decrease in survival was seen in animals treated with either anti-TGF-β mAb or soluble TGF-β receptor.

In still a further example, groups of 10 animals each were treated with a lethal dose of 5-flourouracil along with simultaneous treatment with 0.1-100 mg/day of

an anti-TGF-β mAb or 0.001-10 mg/day of a soluble form of TGF-β receptor. Treatment with either anti-TGF-β mAb or soluble TGF-β receptor resulted in a dose-dependent increase in survival over animals that received 5-flourouracil alone. In addition, examination of bone marrow cells in mice at 2 weeks following 5-fluorouracil treatment showed a dose-dependent increase in cell number after anti-TGF-β mAb or soluble TGF-β receptor treatment.

Other Embodiments

Other embodiments are within the following claims.

Claims

1. A method for expanding growth of hematopoietic stem cells in culture, including a step of admixing a TGF-β antagonist with the culture.

2. The method of claim 1 wherein said TGF-β antagonist comprises a soluble TGF-β receptor.

3. The method of claim 1 wherein the TGF-β antagonist comprises a TGF-β-neutralizing antibody.

4. The method of claim 2 wherein said TGF-β antagonist comprises a TGF-β-neutralizing monoclonal antibody.

5. The method of claim 2 wherein said TGF-β antagonist comprises a plurality of TGF-β-neutralizing antibodies.

6. The method of claim 2 wherein said TGF-β antagonist comprises a monoclonal antibody that neutralizes TGF-β types 1, 2, and 3.

7. The method of claim 2 wherein said TGF-β antagonist comprises a plurality of monoclonal antibodies that in combination neutralize TGF-β types 1, 2, and 3.

8. The method of claim 6 wherein said TGF-β antagonist comprises a monoclonal antibody produced by hybridoma cell line 1D11.

9. The method of claim 1 wherein said TGF-β antagonist is admixed to the culture at substantially the time of initiation of the culture.

10. A method for treating an animal, comprising steps of culturing bone marrow cells, and adding a TGF-β antagonist to the culture to stimulate production of hematopoietic stem cells in the culture, and thereafter administering hematopoietic cells so produced to the animal.

11. The method of claim 10 wherein said stem cells are cells that were derived from an animal of the same species as the animal to be treated.

12. The method of claim 11 wherein said stem cells are cells that were derived the animal to be treated.

13. The method of claim 10 wherein the animal to be treated has an undesirably low amount of hematopoietic cells, or is known or is suspected to be likely to develop an undesirable reduction in amount of hematopoietic cells.

14. The method of claim 10 wherein the animal to be treated is known or is suspected to be likely to develop an undesirable reduction in amount of hematopoietic cells as a result of medical treatment, wherein the step of administering the TGF-β antagonist to the animal is carried out at about the same time as the medical treatment is carried out.

15. The method of claim 10 wherein said TGF-β antagonist comprises a soluble TGF-β receptor.

16. The method of claim 10 wherein said TGF-β antagonist comprises a TGF-β-neutralizing antibody.

17. The method of claim 16 wherein said TGF-β antagonist comprises a TGF-β-neutralizing monoclonal antibody.

18. The method of claim 16 wherein said TGF-β antagonist comprises a plurality of TGF-β-neutralizing antibodies.

19. The method of claim 16 wherein said TGF-β antagonist comprises a monoclonal antibody that neutralizes TGF-β types 1, 2, and 3.

20. The method of claim 16 wherein said TGF-β antagonist comprises a plurality of monoclonal antibodies that in combination neutralize TGF-β types 1, 2, and 3.

21. The method of claim 20 wherein said TGF-β antagonist comprises a monoclonal antibody produced by hybridoma cell line 1D11.

22. The method of claim 10 wherein said TGF-β antagonist is admixed to the culture at substantially the time of initiation of the culture.

23. A method for treating an animal, comprising steps of administering to the animal a TGF-β antagonist.

24. The method of claim 23, wherein the animal to be treated has an undesirably low amount of hematopoietic

cells, or is known or is suspected to be likely to develop an undesirable reduction in amount of hematopoietic cells.

25. The method of claim 23, wherein the animal to be treated is known or is suspected to be likely to develop an undesirable reduction in amount of hematopoietic cells as a result of medical treatment, wherein the step of administering the TGF-β antagonist to the animal is carried out at about the same time as the medical treatment is carried out.

26. The method of claim 23 wherein said TGF-β antagonist comprises a soluble TGF-β receptor.

27. The method of claim 23 wherein the TGF-β antagonist comprises a TGF-β-neutralizing antibody.

28. The method of claim 27 wherein said TGF-β antagonist comprises a TGF-β-neutralizing monoclonal antibody.

29. The method of claim 27 wherein said TGF-β antagonist comprises a plurality of TGF-β-neutralizing antibodies.

30. The method of claim 27 wherein said TGF-β antagonist comprises a monoclonal antibody that neutralizes TGF-β types 1, 2, and 3.

31. The method of claim 27 wherein said TGF-β antagonist comprises a plurality of monoclonal antibodies that in combination neutralize TGF-β types 1, 2, and 3.

32. The method of claim 30 wherein said TGF-β antagonist comprises a monoclonal antibody produced by hybridoma cell line 1D11.

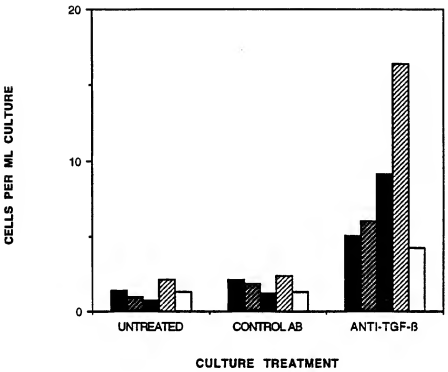
20
10
0
CELLS PER ML CULTURE
UNTREATED
CONTROL AB
ANTI-TGF-ß
CULTURE TREATMENT

Figure 1

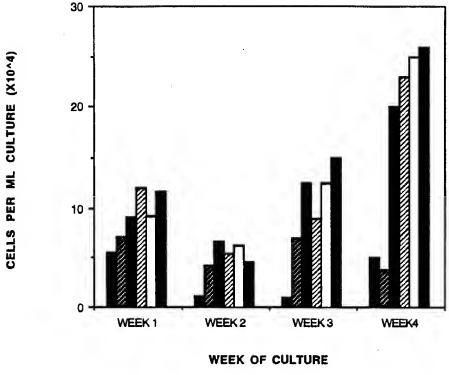
30
20
10
0
CELLS PER ML CULTURE (X10^4)
WEEK 1
WEEK 2
WEEK 3
WEEK4
WEEK OF CULTURE

Figure 2

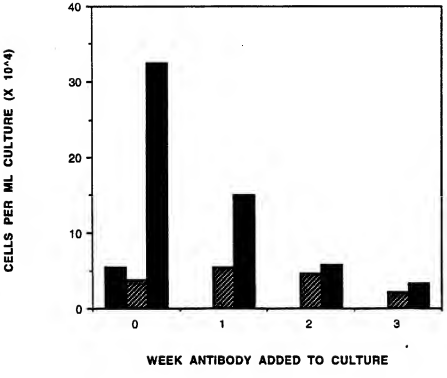
CELLS PER ML CULTURE (X 10^4)
40
30
20
10
0
0
1
2
3
WEEK ANTIBODY ADDED TO CULTURE

Figure 3

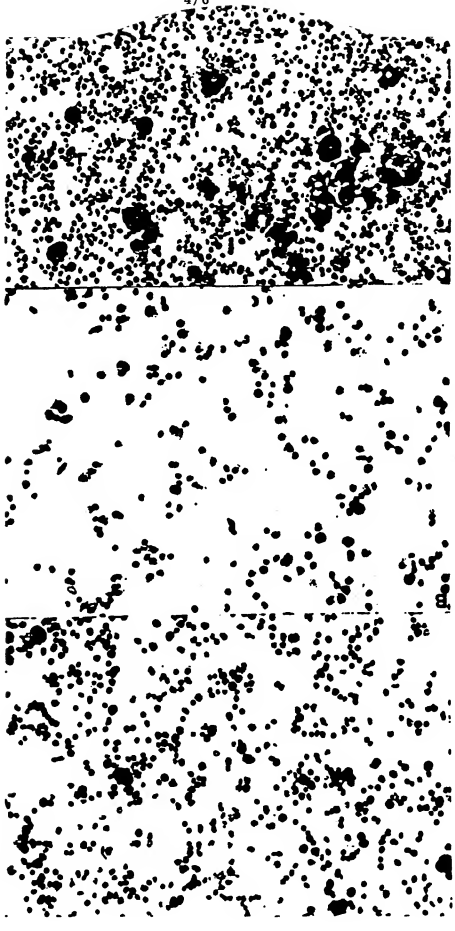

Fig. 4.1 Fig. 4.2 Fig. 4.3

Fig. 5.1 Fig. 5.2 Fig. 5.3

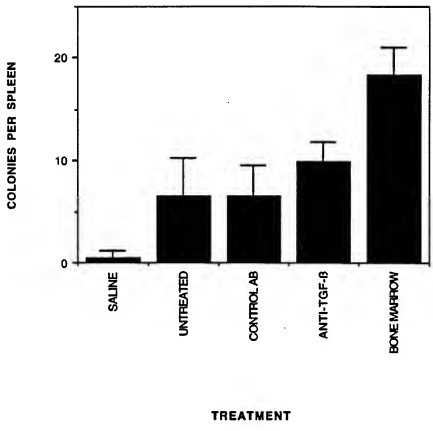
COLONIES PER SPLEEN
20
10
0
SALINE
UNTREATED
CONTROL AB
ANTI-TGF-β
BONE MARROW
TREATMENT

Figure 6

INTERNATIONAL SEARCH REPORT

Int. ational application No.
PCT/US94/01634

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	J. IMMUNOLOGY, VOL. 142, NUMBER 5, ISSUED 01 MARCH 1989, J.R. DASCH ET AL., "MONOCLONAL ANTIBODIES RECOGNIZING TRANSFORMING GROWTH FACTOR BETA", PAGES 1536-1541, ENTIRE DOCUMENT.	1-32
Y	ADVANCES IN CANCER RESEARCH, COLUME 54, ISSUED 1990, M. HERLYN ET AL., "GROWTH-REGULATORY FACTORS FOR NORMAL, PREMALIGNANT, AND MALIGNANT HUMAN CELLS IN VITRO", PAGES 213-234, ESPECIALLY PAGES 230-231.	1-9
Y	J. EXPERIMENTAL MEDICINE, VOL. 174, ISSUED OCTOBER 1991, J. HATZFELD ET AL., "RELEASE OF EARLY HUMAN HEMATOPOIETIC PROGENITORS FROM QUIESCENCE BY ANTISENSE TRANSFORMING GROWTH FACTOR BETA1 OR RB OLIGONUCLEOTIDES", PAGES 925-929, ENTIRE DOCUMENT.	1-32

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be part of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
17 APRIL 1994	02 MAY 1994

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Jill Warden for CHRIS EISENSCHENK
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	CELL, VOL. 67, ISSUED 15 NOVEMBER 1991, X.F. WANG ET AL., "EXPRESSION CLONING AND CHARACTERIZATION OF THE TGF-BETA TYPE III RECEPTOR, PAGES 797-805, ENTIRE DOCUMENT.	1-32
Y	CELL, VOLUME 61, ISSUED 15 JUNE 1990, C.T. JORDAN ET AL., "CELLULAR AND DEVELOPMENTAL PROPERTIES OF FETAL HEMATOPOIETIC STEM CELLS", PAGES 953-963, ENTIRE DOCUMENT.	1-32
X --- Y	J. CELL. BIOCHEM., VOLUME 16(C), ISSUED 1992, W.O. WAEGELL ET AL., "NEUTRALIZING ANTIBODY TO TGF-BETA OVERCOMES NORMAL GROWTH CONTROLS IN LONG TERM BONE MARROW CULTURES", ABSTRACT NUMBER M342, SEE ENTIRE DOCUMENT.	1-9 --- 10-32

INTERNATIONAL SEARCH REPORT

Interna. .al application No.
PCT/US94/01634

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 35/28; C12N 5/08

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/240.25, 70.23; 530/389.2, 388.23; 424/93, 577

B. FIELDS SEARCHED
Minimum documentation searched
Classification System: U.S.

435/240.25, 70.23; 530/389.2, 388.23; 424/93, 577

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

DIALOG, APS, MEDLINE, BIOSYS, EMBASE, LIFESCI